more acidic solutions, with pH < 7·0, a few seconds elapsed before equilibrium was attained. Taking into account the ionization of the haem-linked group on MetMb and the higher oxidation state, the variation of $K_{obs.}$ with pH is shown to confirm the conclusion that 2 moles of H^+ are liberated/mole of acidic MetMb. Using 6·1 for the pK of the group in MetMb as established in other studies, the results give a pK of 7·5 for the group in the higher oxidation state at 20° and $I = 0·04$.

3. The variation of $K_{obs.}$ with temperature gives $\Delta H° = 10·0 \pm 2·0$ kcal./g.mol.: if the ionization of the haem-linked group is allowed for, the value $9·0 \pm 1·0$ kcal./g.mol. is obtained.

4. The dependence of $K_{obs.}$ on ionic strength is in accord with a change in charge from +1 on MetMb to zero on the higher oxidation state.

5. The results are shown to favour the ferryl ion structure, or an isomer of this structure, for the higher oxidation state. The isomeric structures would, in general, require the presence of another ionizing group in myoglobin, but no evidence for such an ionization could be found. With other direct evidence favouring the ferryl ion structure this is to be preferred, and the higher oxidation state may provisionally be named ferrylmyoglobin.

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Tissue Fractionation Studies

6. INTRACELLULAR DISTRIBUTION PATTERNS OF ENZYMES IN RAT-LIVER TISSUE*

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The finding that the acid phosphatase of rat liver is enclosed within a special type of cytoplasmic granules, with sedimentation properties intermediate between those of mitochondria and microsomes, has led to the development of a new scheme of fractionation, whereby enzymes attached to these granules can be readily identified (Appelmans, Wattiaux & Duve, 1955). This scheme has been applied in the present work to the study of the following enzymes, previously shown to resemble acid phosphatase in being unequally distributed between mitochondria and microsomes: DPNH–cytochrome *c* reductase (Hogeboom, 1949; Hogeboom & Schneider, 1950*a*; Strittmatter & Ball, 1954), TPNH–cytochrome *c* reductase (Hogeboom & Schneider, 1950*b*), β-glucuronidase (Walker, 1952), cathepsin (Maver & Greco, 1951), ribonuclease (Schneider & Hogeboom, 1952*b*; Pirotte & Desreux, 1952), deoxyribonuclease (Schneider & Hogeboom, 1952*b*; Kuff & Schneider, 1954), uricase (Schein, Podber & Novikoff, 1951; Schneider & Hogeboom, 1952*a*; Novikoff, Podber, Ryan & Noe, 1953; Kuff & Schneider, 1954) and fumarase (Kuff, 1954). In addition, the distributions of

* Part 5: Appelmans, Wattiaux & Duve (1955).

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rhodanese (Ludewig & Chanutin, 1950) and of succinate–cytochrome *c* reductase (Strittmatter & Ball, 1954), which appear to follow that of cytochrome oxidase, have also been re-investigated.

Most experiments include measurements of cytochrome oxidase, acid phosphatase and glucose-6-phosphatase, each of which is known from earlier investigations to be associated essentially with one of the three groups of cytoplasmic particles so far identified in rat liver (Applemans *et al.* 1955). Following the principle set forth in a recent review (Duve & Berthet, 1954), the distribution of these enzymes has been used as a reference framework serving for the interpretation of the data obtained on other enzymes. Some of the results described here have already been reported in preliminary form (Duve, Gianetto, Appelmans & Wattiaux, 1953; Pressman & Duve, 1954).

EXPERIMENTAL

Tissue fractionations

The experiments were performed on the livers of adult albino rats raised on a standard diet. In most cases, the animals were fasted for 12 hr. before death. The suspension medium was 0·25 M sucrose, usually containing 0·001 M disodium ethylenediaminetetraacetate (Versene). This compound exerts a protective action on glucose-6-phosphatase (Beaufay, Hers, Berthet & Duve, 1954), perhaps also on other enzymes (Slater & Cleland, 1953), and seems to have no undesirable effects.

The animals were killed by a blow on the head and bled, the liver quickly taken out and immersed in a tared beaker containing ice-cold medium. After weighing and allowing a few min. for chilling, the tissue was cut into a few pieces and dispersed with approximately 3 vol. of medium in a homogenizer of the Potter & Elvehjem (1936) type, consisting of a smooth-walled glass tube fitted with a Teflon pestle (Manufactured by Arthur H. Thomas Co, Philadelphia). The tube, kept in cracked ice, was given a single run upward against the rapidly rotating pestle (1300 rev./min.), until all the material had been forced above the latter. The resulting slurry was centrifuged in the cold by 10 000 *g*-min. ($S_{min.}$ = 90 000 *S*).* The sediment, which still contained a large number of unbroken cells in addition to the nuclei, was rehomogenized in about the same quantity of medium and centrifuged by 6000 *g*-min. ($S_{min.}$ = 15 000 *S*). After repeating this operation a second time the nuclear sediment, then almost free of intact cells or gross debris, was redispersed by means of the homogenizer and made up to a final volume usually equal to 4 times the weight of tissue processed, yielding the 1:4 nuclear fraction. The supernatants were combined and made up to volume, to form the 1:10 cytoplasmic extract. The sum of the values obtained separately on the nuclei and cytoplasmic extract was taken as representative of the whole tissue, since mixtures of these two fractions usually undergo extensive agglutination and sometimes yield erratic results.

* The nomenclature used for this description has been explained and justified in previous publications (Duve & Berthet, 1953; Appelmans *et al.* 1955).

The cytoplasmic extract was further fractionated according to the scheme outlined by Appelmans *et al.* (1955), using the Spinco model L preparative ultracentrifuge, with rotor no. 40 ($R_{max.}$ = 8·1 cm., $R_{min.}$ = 4·8 cm.). Three particulate fractions, two mitochondrial and one microsomal, were successively isolated by integrated forces of 33 000 *g*-min. ($S_{min.}$ = 17 300 *S*), 250 000 *g*-min. ($S_{min.}$ = 2300 *S*) and 3 000 000 *g*-min. ($S_{min.}$ = 190 *S*) respectively. As a rule, the sediments were first separated by spinning down 30 or 40 ml. of fluid, collected in a single tube and given two washings in a volume of 10 ml. This was done in order to reduce the dilution of the final supernatant and the time taken for the whole procedure. In each case, the washings were combined with the first supernatant, made up to a known volume and processed for the isolation of the next fraction. The washed granules were finally taken up in small volumes, so as to make the final dilution (expressed in g. original wet tissue/ml.) approximately 1:2 for the mitochondrial fractions and 1:4 for the microsomes. Tared tubes were used and the volumes calculated by dividing the weight of the fraction by 1·05, the estimated density of such suspensions in 0·25 M sucrose at 0°. The dilution of the final supernatant ranged between 1:30 and 1:40.

As described before (Appelmans *et al.* 1955), a special pipette operated by a rubber bulb was used for sucking off supernatants and a micro-homogenizer for resuspending the sediments. The complete fractionation took about 6 hr. to perform. Throughout this time, care was taken to avoid losses and to keep the material as near 0° as possible.

The separation of the two mitochondrial fractions represents the most delicate step in this type of fractionation. The heavy mitochondrial sediment consists of a reasonably well-packed bottom layer of a homogeneous light brown colour, covered by a certain amount of loose material. In separating the supernatant fluid, as much of the upper layer was removed as could be done without disturbing the bottom sediment. The same method was followed after each washing and the final sediment showed only traces of a second layer. The light mitochondrial sediment isolated from the combined supernatants occurs in the form of a small dark-brown pellet, capped by the pink 'fluffy layer'. Most of the latter was removed together with the supernatant fluid and washings, to be finally recovered with the microsomal fraction. Excessive thoroughness in this step sometimes led to the removal of traces of brown material, which could then be detected as a dark spot at the bottom of the red jelly-like microsomal pellet.

Enzyme assays

Hydrolytic enzymes. Acid phosphatase, β-glucuronidase and cathepsin were measured as described previously (Gianetto & Duve, 1955), except that the assays were run at pH 5 for β-glucuronidase and 3·6 for cathepsin. Deoxyribonuclease was measured at 37° in a total volume of 2 ml. containing 3 mg. purified nucleate and 0·1 M sodium acetate buffer pH 5. The reaction was stopped by adding 2 ml. of ice-cold 10% (w/v) perchloric acid. The mixture was centrifuged after standing 10 min. in the cold and the extinction of the supernatant read at 260 mμ. in the Beckman Model DU spectrophotometer. Ribonuclease was assayed in a similar manner, except that 0·25% (w/v) uranyl acetate was added to the perchloric acid used for stopping the

reaction and that the mixture was left for 1 hr. in the cold before centrifuging.

In each of the above cases, total activities were estimated after the enzymes had been completely released by diluting the preparations with distilled water, and subsequently exposing them for 3 min. to the action of a cooled Waring Blendor or for 3 hr. at 37° and pH 5. The latter procedure was preferred after it was found that the Blendor treatment occasionally causes some denaturation of the enzymes, especially of deoxyribonuclease. In one case, the release of β-glucuronidase was assured by running the assays in the presence of 0·1% (v/v) Triton X-100 (Walker, 1952). When free activities were also measured, the assays were run with fresh preparations incubated for 10 min. in the presence of a substrate mixture containing 0·25M sucrose (Gianetto & Duve, 1955). This sugar had no effect on the enzymic activities themselves, except with β-glucuronidase, which is inhibited to the extent of 25–30% by 0·25M sucrose. To make the free and total activities comparable, 0·25M sucrose was also present in the assays of total β-glucuronidase.

Glucose-6-phosphatase, which required no preliminary activation, was assayed at 37° in a total volume of 1 ml. containing 0·04M glucose 6-phosphate, 0·007M histidine and 0·001M ethylenediaminetetraacetate, pH 6·5. The reaction was stopped by the addition of 10% (w/v) trichloroacetic acid and inorganic P determined on the filtrates.

Appropriate blanks were determined in all these assays and subtracted from the observed values. The validity of the methods was verified in control experiments, which showed that the measured activities were proportional to both enzyme concentration and time.

Cytochrome c-linked enzymes. Cytochrome oxidase was assayed by the spectrophotometric method of Cooperstein & Lazarow (1951) in the manner described previously (Appelmans *et al.* 1955).

The DPNH- and TPNH-specific cytochrome *c* reductase activities were measured according to Hogeboom (1949) and Hogeboom & Schneider (1950*a*, *b*), using the Beckman model DU spectrophotometer and cuvettes of 1 cm. light path. The final incubation mixture contained 0·033M potassium phosphate buffer pH 7·4, 0·027M nicotinamide, 2×10^{-4}M sodium cyanide, 4×10^{-5}M oxidized cytochrome *c* and $7{\cdot}5 \times 10^{-5}$M reduced pyridine nucleotide, in a total volume of 3 ml. The change in optical density at 550 mμ. was followed at 15 sec. intervals, the incubation being performed at room temperature. Duplicate measurements were usually made, sometimes at two different levels of enzyme. The reaction rate was reasonably constant in time and proportional to the amount of enzyme added.

Optimum activities were obtained if the cell fractions were first preincubated 30 min. at 0° after dilution in water or 0·001M Versene pH 7·4, and then 2–3 min. at room temperature in the cuvette in the presence of all the components except the reduced nucleotide. The latter was added in a volume of 0·1 ml. to start the reaction. The rate at which the preparation reduced cytochrome *c* in the absence of added nucleotide (endogenous activity) could be measured during the last phase of the pre-incubation. It was generally negligible, except with some preparations of low activity, usually final supernatants. No correction was made for it even in such cases, since it could not be ascertained to what extent pyridine nucleotides were actually involved in the endogenous activity.

The succinate cytochrome *c* reductase activity was assayed in a similar manner, in the presence of 0·033M sodium succinate as electron donor.

Other enzymes. Rhodanese was estimated by the technique of Cosby & Sumner (1945), as modified by Rosenthal, Rogers, Vars & Ferguson (1950). The enzyme was incubated during 2 min. at 20° in a mixture containing 0·066M potassium phosphate buffer pH 7·4, 0·05M sodium thiosulphate and 0·05M potassium cyanide. The reaction was stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid containing 0·15% (w/v) formaldehyde. Thiocyanate was estimated colorimetrically on the filtrates as the ferric complex. The blanks were run as recommended by Rosenthal *et al.* (1950). Activities measured by this method were found to fall off somewhat with increasing enzyme concentration but comparable values could be obtained by running the assays at appropriate levels of enzyme.

Optical tests were used for the assay of fumarase and uricase. It was found advisable to dilute the preparations with 0·005M phosphate buffer pH 7·4 a few min. before the assay, in order to obtain maximal initial rates. The reactions were run at room temperature in cells of 1 cm. light path and followed in the Beckman model DU spectrophotometer. Fumarase was measured according to Racker (1950*a*). The reaction was started by mixing 2 ml. of dilute enzyme with 1 ml. of 0·15M sodium L-malate in 0·15M potassium phosphate buffer pH 7·4. The increase in optical density at 240 mμ. was followed against a blank containing all the components except L-malate. The assay of uricase was performed in a similar manner, by measuring the decrease in optical density at 290 mμ., after adding 2 ml. of dilute enzyme to 1 ml. of a substrate mixture, prepared according to Schneider & Hogeboom (1952*a*) and containing $3{\cdot}75 \times 10^{-4}$M sodium urate and 0·03M potassium phosphate buffer pH 7·4. The rates measured in this manner were constant in time and proportional to the amount of enzyme added.

Materials

Among the substrates used for the enzyme assays, β-glycerophosphate (British Drug Houses Ltd.), L-malate (Eastman-Kodak) and uric acid (Hofmann-La Roche) were commercial samples. The preparations of phenolphthalein glucuronide and haemoglobin have been described before (Gianetto & Duve, 1955). Commercial ribonucleic acid (L. Light and Co.) was purified according to Kunitz (1941), dissolved in dilute NaOH and dialysed exhaustively in the cold against 0·1M acetate buffer pH 5. Deoxyribonucleic acid was isolated from calf thymus by the procedure of Hammarsten (1930) and also dialyzed against 0·1M acetate buffer pH 5. Glucose 6-phosphate was prepared by an enzymic method (Hers, Beaufay & Duve, 1953) and isolated as the crystalline barium salt heptahydrate. Cytochrome *c* was extracted from horse or ox heart according to Keilin & Hartree (1945) or purchased commercially (Sigma Chemical Corp.). DPN, either prepared by the method of LePage & Mueller (1949) or obtained commercially (Schwartz), was subjected to the chromatographic purification procedure of Neilands & Åkeson (1951). The purified products, as assayed with alcohol dehydrogenase, were respectively 88 and 75% pure, without correcting for moisture. TPN was prepared according to LePage & Mueller (1949) and further purified chromatographically by the method of Kornberg & Horecker (1953); it was free of DPN and approximately 90% pure.

Reduction of DPN was performed by dissolving 50 mg. of nucleotide in 5 ml. water, adjusting to pH 9·5 with NaOH and then adding 0·1 ml. ethanol and a suitable quantity of alcohol dehydrogenase, as prepared according to Racker (1950*b*). The reaction was followed with the aid of a glass electrode pH-meter, adding more alkali as needed to maintain the pH at 9·5. After 5 min., when the reaction appeared to have stopped, another 0·1 ml. of ethanol was added and the mixture concentrated *in vacuo*, to remove acetaldehyde. After dilution to the original volume, the whole process was repeated. After the second distillation, the residue was taken up in about 10 ml. water at pH 8·5, quickly heated to 95° and cooled. The denatured enzyme was precipitated by adjusting the pH to 5·5, and the solution filtered and brought to a volume of 25 ml. The product was stored frozen at pH 8 and appeared stable for several months.

A crude preparation of *iso*citric dehydrogenase was used to reduce TPN. The enzyme was extracted from an acetone powder of pig heart and subjected to a single purification step by $(NH_4)_2SO_4$ precipitation, according to Grafflin & Ochoa (1950). A sample of mixed barium *iso*citrate (70%) and citrate (30%), extracted from *Bryophyllum calycinum*, was converted into the free acids by treatment with Amberlite IR-120, and neutralized with NaOH. A quantity of the resulting solution equivalent to 100 μmoles of *d-iso*citrate was allowed to react at pH 8 with 54 mg. of TPN in a vol. of 7 ml. containing $7·6 \times 10^{-4}$ M-$MnSO_4$ and a suitable quantity of *iso*citric dehydrogenase. The reaction was followed by means of the pH meter and the pH maintained at 8 by the addition of alkali. After 1 hr., the mixture was heated to 95°, cooled, acidified to pH 5·5 and filtered. A faint opalescence persisted which could not be removed but did not appear to interfere with the assay. The solution was then adjusted to pH 7·5 and brought to 25 ml. This preparation, which was also kept frozen, tended to reoxidize very slowly on prolonged storage, possibly owing to the catalytic effect of Mn^{2+}. The *iso*citrate used for this preparation was a gift from Dr B. L. Horecker.

Dr F. Strong kindly donated the sample of crystalline antimycin A used in some experiments.

Units and presentation of results

To simplify the construction of tables, the following symbols will be used to designate the isolated fractions: E = cytoplasmic extract; N = nuclear fraction; M = heavy mitochondrial fraction; L = light mitochondrial fraction; P = microsomal fraction; S = final supernatant.

Results of enzyme distribution studies will be expressed in units/g. of original tissue and in per cent of the sum of the activities found in the cytoplasmic extract and in the nuclear fraction.

Except for cytochrome oxidase, one unit of activity refers to the decomposition of 1 μmole of substrate/min. under the conditions of the assay. The molarity of the products of cathepsin action was expressed conventionally in terms of tyrosine equivalents of the colour developed with the Folin & Ciocalteu reagent; that of the breakdown products of nucleic acids in terms of liberated mononucleotides, assuming an average extinction coefficient at 260 mμ. of $8·5 \times 10^6$ cm.^{2} mole^{-1} (Stimson & Reuter, 1945). The other optical constants used were $1·96 \times 10^7$ cm.^{2} mole^{-1} for the change in optical density at 550 mμ. accompanying the reduction of cytochrome *c* (Horecker & Heppel, 1949), $1·22 \times 10^7$ cm.^{2} mole^{-1} for the decrease in optical density at 290 mμ. related to the oxidation of uric acid (Kalckar, 1947) and $2·11 \times 10^6$ cm.^{2} mole^{-1} for the increase in optical density at 240 mμ. caused by the dehydration of malate (Racker, 1950*a*).

As explained elsewhere (Cooperstein & Lazarow, 1951; Appelmans *et al.* 1955), one cytochrome oxidase unit is defined as the amount of enzyme causing the decadic logarithm of the concentration of reduced cytochrome *c* to decrease by one unit/min./100 ml. of incubation mixture.

RESULTS

General survey of quantitative data

Altogether nineteen complete fractionations were performed, involving the simultaneous determination of between four and eleven different enzymic activities. The means and standard deviations of the results obtained in these experiments are listed in Table 1.

The absolute levels of activity recorded in Table 1 resemble those observed by other workers, except for uricase and TPNH cytochrome *c* reductase. Our values for these two enzymes are 4 to 5 times lower than those found by Schneider & Hogeboom (1952*a*) for the uricase of rat and mouse liver, and by Hogeboom & Schneider (1950*b*) for the TPNH cytochrome *c* reductase of mouse liver. Since similar assay conditions were used, these disparities are probably related to differences in the species, strain or maintenance conditions of the animals.

The percentage distributions are in close agreement with those established by means of the classical technique, in which the large granules are isolated in a single fraction, corresponding roughly to the sum of the two mitochondrial fractions separated in the new procedure (Appelmans *et al.* 1955). There is one discrepancy; the nuclear content of cathepsin amounts to about 30% in the experiments of Maver & Greco (1951), and to only a few per cent here. However, these authors themselves attribute their finding to a heavy contamination of the nuclear fraction by large cytoplasmic granules, an interpretation which appears to be supported by the present data.

In most cases, the recoveries range between 90 and 110%. The good recoveries of nitrogen and of more robust enzymes such as acid phosphatase show that no gross errors are introduced by the fractionation procedure itself. Consequently, where discrepancies occur, they must be attributed either to changes undergone by the enzymic systems or to artifacts associated with the assays. In order to reduce the errors introduced by excessive or defective recoveries all the results were recalculated in percentage of the sum of the recovered activities before being used for the various analyses to be described below.

Table 1. *Intracellular distribution of enzymes*

Absolute values are in mg./g. for nitrogen; in units/g. for enzymes. Statistics refer to the means ± the standard deviations. These are given, rather than the standard errors of mean, since they are a measure of individual variability, the most interesting information in the present case. E = cytoplasmic extract; N = nuclear fraction; M = heavy mitochondrial fraction; L = light mitochondrial fraction; P = microsomal fraction; S = final supernatant.

Enzyme	No. of expts.	Absolute values E + N	Percentage values					
			E + N	N	M	L	P	S	Recovery
Nitrogen	19	32·3 ± 4·8	100	13·3 ± 2·6	16·3 ± 4·2	7·4 ± 3·1	24·4 ± 3·3	37·5 ± 5·2	98·9 ± 1·6
Cytochrome oxidase	17	30·6 ± 9·8	100	10·1 ± 5·4	57·7 ± 12	17·4 ± 8·7	3·7 ± 1·6	0	88·9 ± 8·1
Succinate–cytochrome *c* reductase	3	22·9 ± 5·4	100	10·1 ± 6·5	49·1 ± 19	9·9 ± 7·1	17 ± 0·4	1·9 ± 1·9	72·7 ± 15
Rhodanese	3	215 ± 12	100	15·1 ± 1·7	58·2 ± 7·1	23·4 ± 11	3·8 ± 3·7	6·2 ± 5·5	106·7 ± 14
DPNH–cytochrome *c* reductase	6	76·3 ± 32	100	8·4 ± 3·8	19·5 ± 5·0	8·7 ± 2·4	62·1 ± 6·7	5·5 ± 1·7	104·2 ± 4·8
TPNH–cytochrome *c* reductase	3	1·60 ± 0·52	100	10·8 ± 2·4	47·1 ± 16	10·2 ± 7·2	20·8 ± 6·6	16·3 ± 6·8	105·2 ± 12
Fumarase	2	76·3 ± 17	100	8·0 ± 2·6	21·7 ± 7·5	11·0 ± 3·3	28·2 ± 15	22·8 ± 7·0	91·7 ± 9·4
Glucose-6-phosphatase	9	18·2 ± 3·7	100	6·8 ± 2·3	2·7 ± 1·2	7·0 ± 2·0	73·8 ± 9·7	2·7 ± 0·6	92·8 ± 8·7
Acid phosphatase (total activity)	19	6·05 ± 1·3	100	3·6 ± 1·3	24·1 ± 1·6	40·7 ± 6·7	20·1 ± 4·8	13·3 ± 2·9	101·8 ± 5·1
Acid phosphatase (free activity)	12	1·21 ± 0·38	21·7 ± 7·4	2·2 ± 1·1	4·7 ± 2·6	4·6 ± 1·6	10·1 ± 2·7	14·4 ± 2·4	36·0 ± 4·5
Ribonuclease	8	2·70 ± 0·64	100	5·5 ± 1·3	28·3 ± 3·8	33·3 ± 12	11·7 ± 4·4	14·6 ± 5·4	93·4 ± 11·3
Deoxyribonuclease	6	1·31 ± 0·36	100	5·3 ± 1·1	33·6 ± 5·4	31·2 ± 7·7	6·3 ± 2·5	18·9 ± 4·4	95·3 ± 6·5
Cathepsin	9	1·46 ± 0·40	100	4·0 ± 2·0	35·0 ± 11	42·9 ± 9·3	7·5 ± 3·8	17·1 ± 8·3	106·5 ± 12·5
β-Glucuronidase (total activity)	4	0·782 ± 0·11*	100	4·3 ± 1·6	18·8 ± 4·5	23·6 ± 1·4	37·4 ± 4·4	12·0 ± 3·0	96·1 ± 2·0
β-Glucuronidase (free activity)	4	0·284 ± 0·06*	37·2 ± 7·8	2·6 ± 0·8	4·1 ± 2·1	7·8 ± 4·8	28·7 ± 5·6	12·0 ± 3·0	55·2 ± 6·5
Uricase	5	0·290 ± 0·06	100	8·2 ± 2·8	12·8 ± 3·4	47·2 ± 7·3	20·2 ± 7·0	6·5 ± 4·2	94·9 ± 10·5

* Uncorrected for inhibition by sucrose (approximately 28%).

A more illustrative survey of the results recorded in Table 1 is provided by the graphs of Fig. 1, which have been constructed for each group of experiments by plotting the mean relative specific activity of the fractions against their mean relative nitrogen content. The area of each block is thus proportional to the percentage of activity recovered in the corresponding fraction, and its height to the degree of purification achieved over the homogenate. The usefulness of the new fractionation procedure is borne out by the great variety of distribution patterns many of which would hardly be distinguished by the classical technique.

Patterns I, II and III characterize the three reference enzymes, which have been shown in a previous investigation (Appelmans *et al.* 1955) to be associated with distinct groups of cytoplasmic granules: cytochrome oxidase with mitochondria, glucose-6-phosphatase with microsomes, and acid phosphatase with a special group of granules, comparable in size to small mitochondria and possessing the sac-like structure and osmotic properties described in the earlier publications of this series. Pattern I is exhibited also by the succinate cytochrome *c* reductase activity and by rhodanese, pattern III by ribonuclease, deoxyribonuclease, cathepsin and, in somewhat modified form, β-glucuronidase. Fumarase and the TPNH- and DPNH-specific cytochrome *c* reductase activities show complex partitions, apparently combining features of patterns I and II. The distribution of uricase characterizes a special pattern (IV).

Detailed analysis of results

Succinate cytochrome c *reductase and rhodanese.* According to the results averaged in Fig. 1, both activities have distributions very similar to that of cytochrome oxidase, suggesting that they have the same intracellular location as this enzyme and are likewise confined exclusively to the mitochondria. This conclusion is strengthened by a closer examination of the results, which shows that the relative content of every individual fraction in succinate cytochrome *c* reductase or rhodanese is equal, within experimental errors, to its relative level of cytochrome oxidase. This correlation is illustrated by Fig. 2.

The observed association between the two components of the succinoxidase system is in agreement with the results of Strittmatter & Ball (1954), and consistent with the earlier investigations showing that this system has the same distribution as cytochrome oxidase (Schneider & Hogeboom, 1950) and can be isolated as an entity by more drastic procedures than those used in differential centrifuging (Keilin & Hartree, 1947; Ball & Cooper, 1949). It should, however, be pointed out that the recovery of the reductase system is very defective, averaging

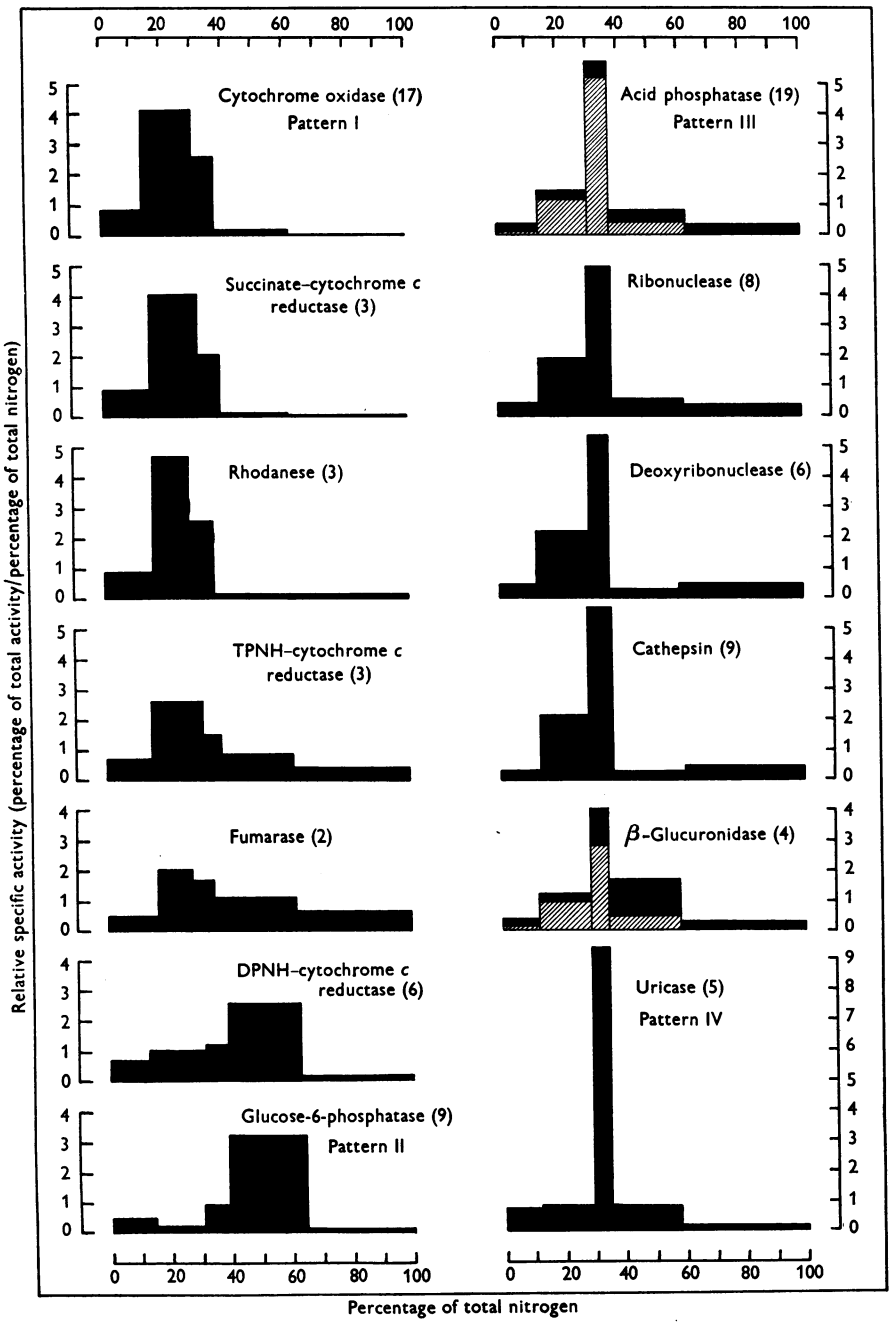

Fig. 1. Distribution patterns of enzymes. Ordinate: mean relative specific activity of fractions. Abscissa: fractions are represented by their relative nitrogen content, in the order in which they are isolated, i.e. from left to right: N, M, L, P and S (see Table 1). Shaded areas represent bound activities (total minus free activities). For description of patterns I–IV see text. Numbers in brackets refer to number of expt.

only about 73 % in our experiments. This could be accounted for by the observations of Schneider & Hogeboom (1950), who demonstrated the presence of an activator of the succinoxidase system in a cytoplasmic extract from which the mitochondria had been removed.

DPNH- and TPNH-cytochrome c *reductases.* These activities exhibit diffuse distributions, which do not coincide with any of the reference patterns (Fig. 1). The TPNH-specific activity is largely concentrated in the mitochondrial fractions, with a distribution similar to that of cytochrome oxidase, but comes down also to a significant extent with the microsomes. On the other hand, the DPNH-specific activity, although mainly microsomal, occurs in the heavier fractions in a proportion too high to be explained by mere contamination. Even the final supernatants seem to be fairly active, especially when TPNH is the electron donor. However, as pointed out in the section on techniques, the assays on the final supernatants were complicated by their endogenous activity. The latter was of the same magnitude as the change of optical density occurring after addition of TPNH, so that a considerable error may have been made in the estimate of the true TPNH-specific activity. With the more active DPNH-specific system this error was smaller but not negligible.

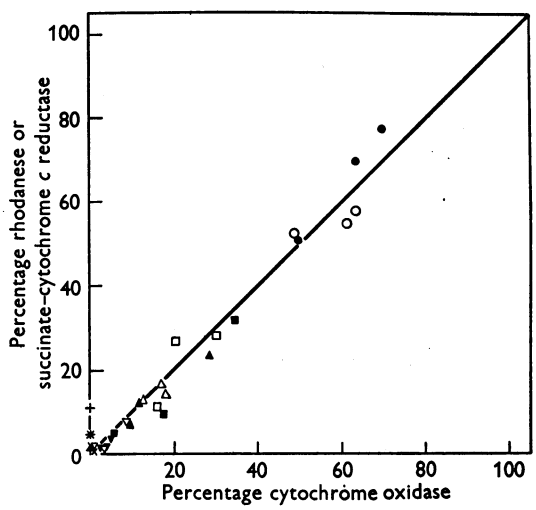

Fig. 2. Correlation between succinate–cytochrome *c* reductase, rhodanese and cytochrome oxidase. Reductase: ▲, N; ●, M; ■, L; ▼, P; ×, S. Rhodanese: △, N; ○, M; □, L; ▽, P; +, S. For explanation of symbols N, M, L, P and S see text and Table 1.

With respect to the more reliable values obtained on the four particulate fractions, the question arises whether they reflect unimodal or bimodal distributions. Supporting the second interpretation are the following calculations, testing the assumption that the observed distributions are simple combinations of patterns I and II. Let a and b be the percentages of activity following the distribution of cytochrome oxidase and glucose-6-phosphatase respectively, R, O and P the percentages of reductase, oxidase and phosphatase actually found in a given fraction. Then the assumption made is expressed by the following equation, which should be applicable to each fraction:

$$100R = aO + bP. \quad (1)$$

Values for a and b were computed in each experiment from the data obtained on fractions M and P. For these fractions, therefore, the experimental values coincide by definition with those calculated by means of Eqn. 1. The check is provided by calculating values for the other fractions and for the total $(a+b)$ and comparing them against the experimental data. As shown in Table 2, the values of a and b are reasonably constant from one experiment to the other, their sum is equivalent to the total percentage of enzyme found in particulate form and a good fit obtains in almost every case between the

Table 2. *Bimodal distribution of cytochrome* c *reductases*

For method of calculation and meaning of symbols, see text.

Electron donor	Expt. no.	a	b	$a+b$	% in N		% in L		% in S	
					Found	Calc.	Found	Calc.	Found	Calc.
DPNH	1	26·9	72·1	99·0	5·8	7·5	9·7	10·8	5·5	0·7
	2	26·0	68·0	94·0	13·2	12·0	5·3	6·2	7·5	1·9
	3	20·2	79·2	99·4	5·9	6·5	11·8	11·7	3·2	2·1
	4	24·1	68·1	92·2	10·8	5·6	8·2	9·5	6·1	2·2
	5	30·4	70·1	100·5	5·6	7·7	6·6	6·5	3·4	2·0
	6	21·6	75·7	97·3	5·6	7·9	8·5	8·3	6·3	1·5
	Mean	**24·9**	**72·2**	**97·1**	**7·8**	**7·9**	**8·4**	**8·8**	**5·3**	**1·7**
TPNH	1	68·6	29·7	98·3	7·9	21·7	4·2	6·0	17·9	1·3
	2	63·8	15·6	79·4	11·0	8·2	19·3	22·8	21·7	0·4
	3	71·4	17·8	89·2	12·1	7·6	6·9	7·6	7·5	0·5
	Mean	**67·9**	**21·0**	**88·9**	**10·4**	**12·5**	**10·1**	**12·1**	**15·7**	**0·7**

calculated and the experimental values for fractions N and L. The activities found in the final supernatant are regularly higher than predicted by the hypothesis, a fact which could be explained either by the presence of some soluble enzyme, or, as pointed out above, by errors in the assays.

Direct evidence in support of the duality of cytochrome *c* reductases is provided by comparison of the properties of the mitochondrial and microsomal systems. Such experiments were performed on carefully washed heavy mitochondrial fractions, almost free of microsomes (containing less than 1 % of the total glucose-6-phosphatase activity) and on microsomes, isolated after centrifuging down all the large granules by 250 000 *g*-min., and discarding them together with the superimposed fluffy layer.

The results of Table 3 show that pretreatment of mitochondria with distilled water causes a significant enhancement of their apparent cytochrome *c* reductase activities and that the corresponding microsomal systems are entirely unaffected by the water treatment. It thus appears that the structural barrier which restricts the activity of the mitochondrial systems (Lehninger, 1951), is absent in the microsomes.

Table 3. *Influence of treatment with distilled water on cytochrome* c *reductase activities*

The fractions were kept for 30 min. at 0° in 0·25 M sucrose or distilled water, and then assayed.

	Cytochrome *c* reductase (units/g.)			
	M fraction		P fraction	
Electron donor	Sucrose	Water	Sucrose	Water
Succinate	1·4	6·9	—	—
DPNH	9·2	15·0	41·2	41·6
TPNH	0·21	0·54	0·30	0·29

Even more significant differences are brought to light by the action of antimycin A, an antibiotic which has been shown to inhibit the succinoxidase system, presumably by blocking Slater's factor (Ahmad, Schneider & Strong, 1950; Potter & Reif, 1952). Fig. 3 illustrates the results of an experiment in which the three cytochrome *c* reductase activities were measured on mitochondria pre-incubated for 1 hr. in distilled water containing increasing quantities of antimycin A. It is seen that the DPNH- and TPNH-specific activities measured under these conditions were partly dependent on an antimycin A-sensitive factor, showing the same titration curve as the factor involved in succinoxidase. On the other hand, the cytochrome *c* reductase activities of microsomal preparations were found to be entirely insensitive to antimycin A, even when this inhibitor was added in amounts greatly exceeding those which were effective on the mitochondrial systems.

These results, which will be elaborated upon in a subsequent publication, are in agreement with the observation of Potter & Reif (1952) that the DPNH–cytochrome *c* reductase of whole liver dispersions is inhibited only to the extent of 25 % or less by antimycin A. They are further supported by the data of Strittmatter & Ball (1954) and Chance & Williams (1954), who have shown that

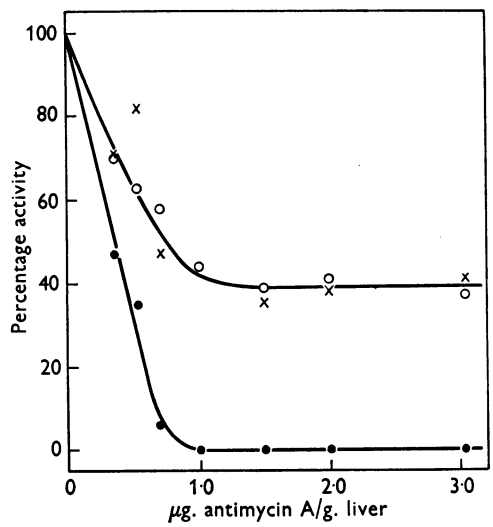

Fig. 3. Effect of increasing amounts of antimycin A on cytochrome *c* reductase systems. Washed M fraction originally 1:4, diluted 40 times with distilled water containing antimycin A at the concentration shown on the abscissa, and the assays carried out under standard conditions. For DPNH- and succinate–cytochrome *c* reductases, 0·1 ml. of enzyme was used per cuvette; for TPNH–cytochrome *c* reductase, 0·8 ml. of enzyme. ●, Succinate; ○, DPNH; ×, TPNH.

the transfer of electrons from DPNH to cytochrome *c* is mediated in liver microsomes by cytochrome b_5 or *m*, a pigment which has been found specifically located in these particles (Strittmatter & Ball, 1952, 1954). A cytochrome b_5-linked system has also been described in *Cecropia* midgut and likewise differentiated from another reductase present in flight muscle, by its insensitivity to antimycin A (Pappenheimer & Williams, 1954; Chance & Pappenheimer, 1954). As shown in the present investigation, a similar duality obtains in the case of the TPNH–cytochrome *c* reductase.

It is concluded from these various results that the distribution patterns observed for the two cytochrome *c* reductase activities reflect the existence of two distinct pairs of systems, located respectively in the mitochondria and in the microsomes.

Fumarase. The distribution of this enzyme resembles that of the TPNH–cytochrome *c* re-

ductase, except that a larger proportion of activity is found in the final supernatant. As shown in Table 4, the results obtained on the four particulate fractions are also compatible with a bimodal distribution obeying Eqn. 1. The calculated amount of mitochondrial enzyme (a) is practically the same in the two experiments but the microsomal values (b) are very different. It should be pointed out in this respect that the microsomes were unwashed in Expt. 1 and washed twice in Expt. 2. It seems therefore that washing can effectively remove fumarase activity from the microsomes. This fact suggests that the extra-mitochondrial enzyme, which accounts for 60% of the total activity, has a single intracellular location and that its unequal distribution between the microsomes and the final supernatant is the result of an artifact. Either the enzyme is truly microsomal but easily washed off from these particles, or it belongs to the non-particulate fraction of the cell, and is secondarily adsorbed on the microsomes. The latter explanation is supported by the results of Kuff (1954), who showed that mouse-liver fumarase is strongly adsorbed by microsomes, but not by mitochondria.

activity at pH 5. By applying the methods used in the study of the other hydrolases, it was found that mitochondrial fractions exhibit low nuclease activities in an assay of 10 min. duration performed in the presence of 0·25 M sucrose, and are activated approximately tenfold by treatment in a Waring Blendor, exposure to distilled water or to a surface active agent (Triton X-100), incubation at pH 5 and 37° for 3 hr., repeated freezing and thawing, etc. Both enzymes were found to be essentially in soluble form in such activated preparations. In view of these facts, it was decided to repeat on the two nucleases the comparative experiments previously carried out on the other enzymes (Gianetto & Duve, 1955).

Graded activation of the granules was elicited by a variety of procedures, and the release of the nuclease and of acid phosphatase was studied simultaneously. The techniques were identical with those of Gianetto & Duve (1955), except that a combined substrate mixture was used, containing both nucleate and β-glycerophosphate. The assays were run as described in the section on techniques for the free nuclease activities and the inorganic P

Table 4. *Bimodal distribution of fumarase*

For method of calculation and meaning of symbols, see text.

Expt. no.	a	b	$a+b$	% in N		% in L		% in S	
				Found	Calc.	Found	Calc.	Found	Calc.
1	41·6	44·3	85·9	6·2	8·3	8·9	9·8	18·1	1·1
2	38·5	24·3	62·8	9·5	9·3	15·9	13·0	32·5	0·4
Mean	**40·1**	**34·3**	**74·4**	**7·9**	**8·8**	**12·4**	**11·4**	**25·3**	**0·8**

Ribonuclease, deoxyribonuclease, cathepsin and β-glucuronidase. In addition to showing distribution patterns similar to that of acid phosphatase (Fig. 1), these four hydrolases also share the peculiar structural properties of this enzyme. This has been demonstrated previously for β-glucuronidase and cathepsin (Gianetto & Duve, 1955) and has now been observed with the two nucleases as well.

It had already been shown by Schneider & Hogeboom (1952*b*) that the ribonuclease and deoxyribonuclease of the large granules are released in soluble form by treatment with sonic vibrations, with a concomitant 35–50% increase in their overall activity. Pirotte & Desreux (1952) have also described the activation of liver ribonuclease, following a number of treatments. However, their assays were performed at pH 7·3 and appeared to be complicated by inhibitory effects, which were suppressed by exposure of the tissue preparation to 0·25 N-H_2SO_4. In the present studies, this treatment was indeed found to cause a considerable increase of the activity at pH 7·3, but to lower somewhat the

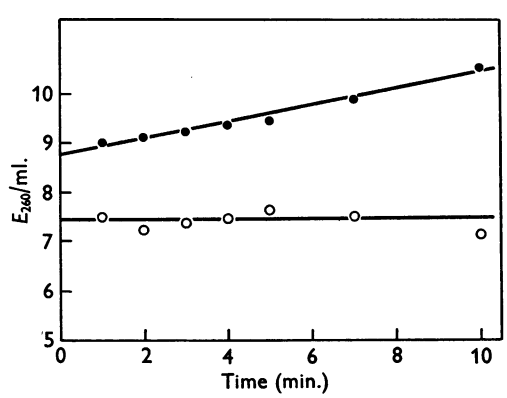

Fig. 4. Kinetics of free ribonuclease activity. Cytoplasmic extract incubated at 30° with substrate and denatured at time shown (●); or incubated alone, denatured at time shown and then mixed with substrate (○). Ordinates give the u.v. absorption of the filtrates, referred to 1 ml. of the original mixture, which contained 2·5 mg. ribonucleic acid and 50 mg. of tissue/ml.

liberated by the phosphatase was measured on part of the filtrate. A short time of contact between substrate and undenatured tissue preparation was found to be necessary to obtain a true ribonuclease blank. If the preparation was first denatured and then mixed with the substrate, the u.v. absorption of the filtrate was distinctly lower than the value obtained by extrapolation from kinetic studies (Fig. 4). Accordingly, free ribonuclease activities were measured by subtracting the values obtained after incubating for 2 and 10 min. The assays of total ribonuclease, which were run with much smaller amounts of enzyme, were not significantly complicated by this artifact. It did not occur in the measurements of deoxyribonuclease.

In Figs. 5 and 6 are plotted the free nuclease activities of variously treated mitochondrial preparations, as a function of the simultaneously measured free acid phosphatase activities. These results show that ribonuclease and deoxyribonuclease, like β-glucuronidase and cathepsin, are released from the granules in a manner which strikingly resembles, both qualitatively and quantitatively, the mode of liberation of acid phosphatase.

The significance of these similarities is somewhat obscured by the observation that the distribution

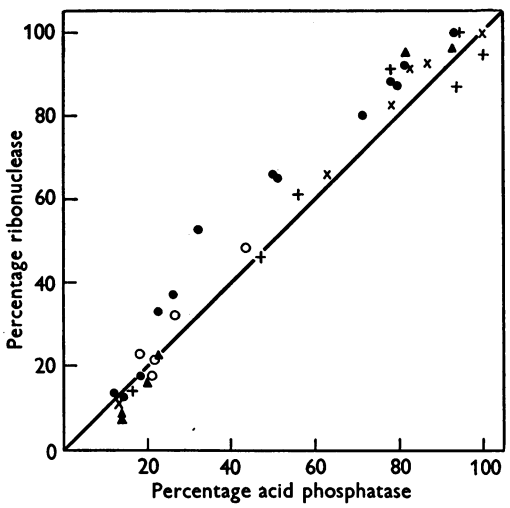

Fig. 5. Parallel release of ribonuclease and acid phosphatase. Free enzymic activities of granules subjected to decreasing sucrose concentrations (●), or to increasing amounts of Triton X-100 (▲), frozen and thawed an increasing number of times (+), exposed to a Waring Blendor (×) or incubated at pH 5 and 37° (○) for increasing lengths of time. All results are expressed in percentage of the highest observed activity.

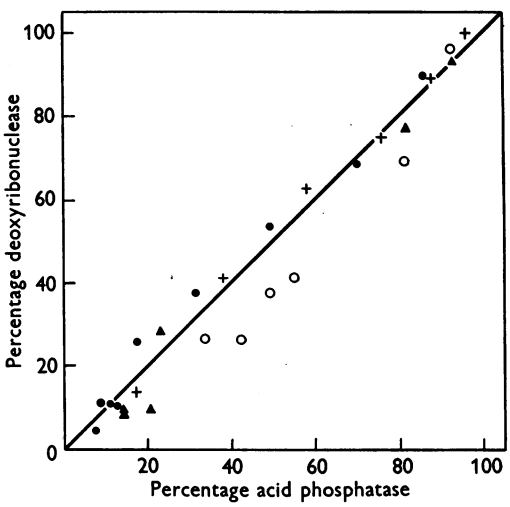

Fig. 6. Parallel release of deoxyribonuclease and acid phosphatase. Free enzymic activities of granules subjected to decreasing sucrose concentrations (●), or to increasing amounts of Triton X-100 (▲), frozen and thawed an increasing number of times (+) or incubated at pH 5 and 37° for increasing lengths of time (○). All results are expressed in percentage of the highest observed activity.

Table 5. *Relative content of enzymes in isolated fractions*

Differences were computed in each individual experiment and averaged. Means are accompanied by their standard error. APase = acid phosphatase; RNase = ribonuclease; DNase = deoxyribonuclease; Cat = cathepsin; β-Gase = β-glucuronidase. For other symbols see text and Table 1.

No. of expts.	Differences	N	M	L	P	S
8	% RNase − %APase	3·0±0·38***	7·2±1·7**	−8·1±3·9	−3·8±1·3*	1·7±1·9
6	% DNase − % APase	2·7±0·45**	11·1±1·9**	−11·1±1·6***	−9·0±1·0***	6·3±1·2**
9	% Cat − % APase	0·6±0·3	8·9±1·0***	−2·1±1·8	−9·9±0·8***	2·5±2·8
4	% β-Gase − % APase	1·6±0·57	−2·5±0·8	−15·9±2·2**	18·4±1·1***	−1·6±2·1
6	% DNase − % RNase	−0·2±0·4	1·9±2·1	1·8±3·0	−5·9±2·1*	2·4±0·9*
8	% Cat − % RNase	−2·3±0·53**	2·4±2·5	5·2±4·7	−6·0±1·3**	0·7±3·2
6	% Cat − %DNase	−2·5±0·53**	−1·6±3·3	8·2±2·9*	−0·8±0·9	−3·3±4·9

* Significant at level of $P < 0{\cdot}05$.
** Significant at level of $P < 0{\cdot}01$.
*** Significant at level of $P < 0{\cdot}001$.

patterns of the five enzymes are only roughly parallel and show small but significant differences, when analysed individually. As evidenced by the results of Table 5, no pair of enzymes shows an entirely identical partition amongst the five isolated fractions. The closest similarity is found between the two nucleases, whereas β-glucuronidase differs most markedly, being considerably more concentrated in the microsomes than any of the other hydrolases. This point was given special attention.

The possibility of an artifact was explored in two different manners. By running the assays of total activity with and without 0·25 M sucrose, it could be verified that the degree of inhibition by this sugar does not vary from one fraction to the other, being approximately 28 % in all cases. In another experiment, the possible involvement of the inhibitor described by Walker & Levvy (1953) was investigated by performing the assays of total activity in the presence and absence of 0·075 % (v/v) Triton X-100, a non-ionic detergent which has been shown by these authors to suppress the observed inhibition. Identical values were obtained on all the fractions. Inhibitions were only observed on aged preparations and on Blendor-treated mitochondrial suspensions which had been further clarified by high-speed centrifuging. Addition of the insoluble residue to the soluble fraction, which contained most of the activity and about 25 % of the total nitrogen, caused a marked inhibition which was reversed by Triton X-100. The pH/activity curve of the enzyme was also greatly modified by the inhibitor, which seemed to arise from damaged mitochondrial fragments.

As shown in Table 1, the difference between β-glucuronidase and the other hydrolases shows up even in the initial extracts, which contain about 40 % free glucuronidase, whereas the proportion of free activity is only 20 % either for acid phosphatase (Table 1) or, as was found in other experiments not reported in the present paper, for the two nucleases. This excess free activity comes down with the microsomes, as is clearly shown by the distribution pattern (Fig. 1). These facts suggest the existence of two distinctly located β-glucuronidases, a possibility which is supported by the findings of Mills, Paul & Smith (1953) and of Smith & Mills (1953). The situation with β-glucuronidase would thus be analogous to that observed with 'arylsulphatase' (Dodgson, Spencer & Thomas, 1955) and with 'acid phosphatase', when measured with phenyl phosphate (Tsuboi, 1952), a substrate which is also acted upon by glucose-6-phosphatase (Beaufay & Duve, 1954). Supporting this interpretation are the results described in Fig. 7, which show that the microsomal activity has a pH/activity curve different from that of the soluble enzyme extracted from mitochondrial preparations.

The existence of several glucuronidases has however been denied by a number of authors, who have put forward the view that the components which have been separated are complexes of the same enzymic protein with different tissue constituents (see Levvy, 1953). Of particular interest are the observations of Bernfeld & Fishman (1950) and Smith & Mills (1953) showing that purified β-glucuronidase is strongly activated by nucleic acids and that combination of the enzyme with various compounds of high molecular weight may displace the pH-optimum above 5. The excess activity found in free form in the extracts and in the microsomal fraction may very well be due to phenomena of this sort, since microsomes contain the largest part of the cell's ribonucleic acid and the assays are run at pH 5.

At the present time, it is not possible to choose between these alternative explanations. Whichever one is valid, it is clear that after correction for the excess microsomal activity the resulting distribution pattern becomes much more similar to that of the other hydrolases.

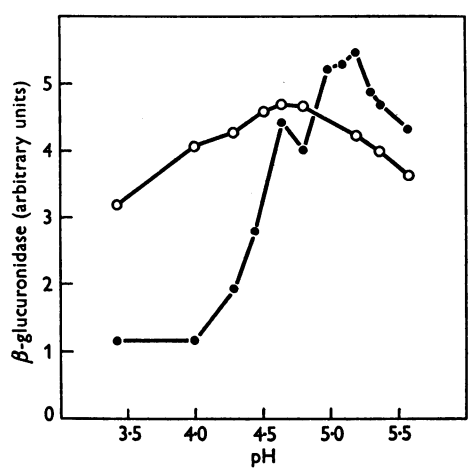

Fig. 7. Influence of pH on β-glucuronidase activity in mitochondria and microsomes. Blendor-treated M+L fraction clarified by high-speed centrifuging (○) and P fraction (●). Ordinates are arbitrary.

Table 6. *Relative content of uricase and acid phosphatase in isolated fractions*

For details and symbols see Table 5.

No. of expts.	Difference	N	M	L	P	S
5	% Uricase − % APase	5·8±1·0**	−10·7±2·6*	7·2±3·7	4·6±3·2	−6·9±1·9*

Uricase. This enzyme is also concentrated in fraction L, where its specific activity is particularly high, regularly showing an approximately tenfold increase over that of the original tissue. It differs from acid phosphatase, and even more from the related hydrolases, by being less abundant in fraction M, more so in fractions L and P (Table 6).

DISCUSSION

According to their distribution patterns, the enzymes studied can be divided in four groups, which are believed to correspond to at least three distinct classes of cytoplasmic granules.

The first group comprises cytochrome oxidase, succinate–cytochrome *c* reductase, rhodanese, 40 % of the total fumarase and the two antimycin A-sensitive pyridinocoenzyme–cytochrome *c* reductase systems, the latter being responsible for an average of 25 % of the total activity specific for DPNH and of 68 % of that specific for TPNH. All these enzymes have been found to be associated, together with a substantial part of the cell nitrogen, with the heavier cytoplasmic granules, and may therefore be considered as truly mitochondrial. It is thus confirmed by a more specific technique that the mitochondria contain a full complement of catalysts necessary for electron transport, in agreement with current ideas concerning the role of these bodies in cellular oxidations.

With the present scheme of fractionation, the mitochondria are distributed unevenly over four different fractions. It is of some significance that this subfractionation has not brought to light any evidence of enzymic heterogeneity in the granules at least as far as the above-mentioned activities are concerned. This conclusion is supported directly by the correlations observed between cytochrome oxidase, succinate–cytochrome *c* reductase and rhodanese (Fig. 2), indirectly by the fact that the observed distributions of the other enzymes could be expressed in a satisfactory manner by a system of equations, based implicitly on the assumption that granules of a given population are enzymically homogeneous, or at least cannot be separated by centrifuging into subgroups differing significantly in relative enzymic content.

In the second group, which comprises the microsomal enzymes, are found glucose-6-phosphatase, the antimycin A-insensitive pyridinocoenzyme–cytochrome *c* reductase systems (and cytochrome b_5, which is probably associated with these systems), possibly also part of the fumarase activity as well as a special β-glucuronidase, maximally active at pH 5·2–5·3 and representing about 20 % of the total activity. Although equations based on the assumption of enzymic homogeneity have been shown to be successfully applicable in most of these cases, undue significance should not be attached to this fact, since the microsomes are concentrated to a large extent in a single fraction and occur as mere contaminants in the others. The possible function of electron-transferring enzymes in the microsomes raises interesting questions, which cannot be answered at the present time.

The third group of enzymes includes acid phosphatase, ribonuclease, deoxyribonuclease, cathepsin and 80 %, if not all, of the β-glucuronidase activity. As shown in a previous publication (Appelmans *et al.* 1955), there are strong grounds for the belief that the peculiar distribution of acid phosphatase reflects the existence of a distinct class of granules and the finding, recorded above, that mitochondria appear to be homogeneous with respect to a number of enzymes provides additional support for this interpretation. The fact that the other enzymes in this group are dissociated from cytochrome oxidase almost as markedly as acid phosphatase, and show distribution patterns very similar to that of the latter enzyme, justify the provisional conclusion that they belong to granules of the same class. For practical purposes, it is proposed to refer to these granules as lysosomes, thus calling attention to their richness in hydrolytic enzymes.

According to the data reported in this and in a previous paper (Gianetto & Duve, 1955), the five hydrolases of this group are released in a parallel fashion in preparations subjected to graded activation by means of a variety of procedures, suggesting strongly that lysosomes form a single population of enzymically homogeneous granules. The systematic differences in distribution which were uncovered (Table 5) are not in accord with this interpretation, but do not suffice to invalidate it. They are relatively small in absolute magnitude and could be due to artifacts. It is necessary to point out in this respect that the exact distribution pattern of enzymes of this type is determined not only by the partition of the granules in which they are contained, but also by the manner in which the free activities, which represent more than one-third of the total activities at the end of the fractionation, become redistributed amongst the fractions as a result of adsorption and other phenomena. Another complication arises from the fact that the fairly drastic procedures required to release the enzymes before assaying may have caused selective denaturation. Finally, in addition to the ordinary difficulties attending the measurement of enzymes in complex media, one must still consider the possible existence of subtler interactions or the involvement of more than one enzyme species, as was shown in the case of β-glucuronidase. All these causes of error are minimized in the comparative activation experiments, which should therefore be regarded as having greater diagnostic significance. However, unless the

conflicting evidence of the distribution experiments can be accounted for, one may not rule out the alternative possibilities that lysosomes are either moderately heterogeneous in their enzymic content or consist of several species.

The solution of these problems, as of those raised by the cytological nature and function of lysosomes, must await the isolation of these granules in the pure state. Their enzymic equipment suggests that they may be concerned with localized phenomena of acid intracellular digestion and have little to do with the major metabolic processes. They could possibly be related to the mitochondria-like bodies, which were shown by Horning (1926) to be involved in the formation of digestive vacuoles in amoeba. There is little doubt that their release and breakage is the main factor responsible for the autolysis which takes place in tissue dispersions and mitochondrial preparations. The beneficial effects of a medium made isotonic with sucrose on the preservation of mitochondrial functions would bear reexamining with this fact in mind.

The distribution observed for uricase suggests that this enzyme belongs either to lysosomes or to yet another class of granules. Since uricase is essentially insoluble and might be expected to sediment with the 'ghosts' of damaged particles as well as with intact granules, it is difficult to distinguish between these two possibilities. However, the relatively high activities found in the nuclei, the low values observed in the heavy mitochondrial fraction, as well as the general shape of the distribution pattern all suggest that uricase is associated with a special group of granules, resembling large microsomes rather than small mitochondria, and differing from lysosomes by a greater uniformity in sedimentation properties. Of interest in this respect are the results of Novikoff, Podber, Ryan & Noe (1953), who have identified a species of large microsomes, which were concentrated in uricase-rich fractions. Slautterback (1952) has also described several varieties of microsomes by means of electronmicroscopy.

The activities found in the final supernatant were negligible or relatively small for most of the insoluble enzymes studied. As pointed out above, the TPNH–cytochrome *c* reductase activity may have been overestimated in this fraction. Soluble enzymes of the lysosome group are all present in the supernatant to the extent of 10–20 %. Part of these activities undoubtedly originate from damaged granules, but it is possible that small amounts of enzyme pre-exist in soluble form in the intact cell. This might be expected to occur, if, as suspected, lysosomes should be concerned with the local secretion of hydrolytic enzymes. The problem raised by the presence of fumarase in the final supernatant (and in the microsomes) has already been discussed. In view of the results obtained by Kuff (1954) and in the present work, the most probable interpretation is that the extra-mitochondrial fraction of this enzyme, i.e. 60 % of the total activity, belongs to the diffusible elements of the cytoplasm but is easily adsorbed by the microsomes.

In conclusion, it may be stated that the results obtained tend to validate the assumptions underlying the use of reference enzymes, namely that specific enzymic species have single intracellular locations and that granules of a given class are enzymically homogeneous (Duve & Berthet, 1954). Some conflicting evidence has however also been brought to light, which unless attributable to artifacts, indicates the existence of greater complexities.

SUMMARY

1. The intracellular distribution of a number of enzymes has been investigated in rat liver according to a new fractionation scheme, in which the classical mitochondria are divided into two subfractions. The observed distribution patterns were compared against those of cytochrome oxidase, acid phosphatase and glucose-6-phosphatase, which served as reference enzymes. The data were interpreted in the light of the information furnished by these comparisons and by additional experiments.

2. The distribution patterns of succinate cytochrome *c* reductase and of rhodanese followed that of cytochrome oxidase, and it was concluded that these systems likewise belong to the mitochondria.

3. The DPNH– and TPNH–cytochrome *c* reductase activities showed complex distributions, reflecting the existence of two distinct pairs of systems, associated respectively with the mitochondria and the microsomes and differing in their susceptibility to activation by distilled water and inhibition by antimycin A.

4. About 40 % of the total fumarase activity was found in the mitochondria, and the remainder recovered partly in the final supernatant, partly in the microsomes, possibly in the latter case as the result of an adsorption artifact.

5. Ribonuclease, deoxyribonuclease, cathepsin and the major part of β-glucuronidase showed distributions analogous to that of acid phosphatase. In addition, they resembled this enzyme in being practically unreactive towards their respective substrates in intact preparations, and were released in a parallel fashion from granules subjected to graded activation by a variety of means. These results were taken to indicate that the four hydrolases belong to the same distinct group of granules previously shown to contain acid phosphatase. The name lysosomes has been proposed for these granules.

6. The microsomes contained a 20% excess of β-glucuronidase, which differed from the remaining activity by a less acid pH-optimum. A second enzyme species appears to be involved, but the possibility of an interaction between adsorbed enzyme and ribonucleic acids cannot be ruled out.

7. Uricase exhibited a unique distribution, indicating that this enzyme is attached either to the insoluble framework of lysosomes or to a fourth distinct group of granules with the properties of large microsomes.

8. In discussing these results, it is pointed out that many afford direct support to the guiding assumptions that distinct enzymic species have single intracellular locations and that granules of a given class are enzymically homogeneous. Some conflicting evidence has also been brought to light.

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